Human Immunodeficiency Virus Type 1 Viral Protein R Localization in Infected Cells and Virions

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The subcellular localization of human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr) was examined by subcellular fractionation. In HIV-1-infected peripheral blood mononuclear cells, Vpr was found in the nuclear and membrane fractions as well as the conditioned medium. Expression of Vpr without other HIV-1 proteins, in two different eukaryotic expression systems, demonstrated a predominant localization of Vpr in the nuclear matrix and chromatin extract fractions. Deletion of the carboxyl-terminal 19-amino-acid arginine-rich sequence impaired Vpr nuclear localization. Indirect immunofluorescence confirmed the nuclear localization of Vpr and also indicated a perinuclear location. Expression of Vpr alone did not result in export of the protein from the cell, but when coexpressed with the Gag protein, Vpr was exported and found in virus-like particles. A truncated Gag protein, missing the p6 sequence and a portion of the p9 sequence, was incapable of exporting Vpr from the cell. Regulation of Vpr localization may be important in the influence of this protein on virus replication.

The human immunodeficiency virus type 1 (HIV-1) genome is more complex than those of murine and avian retroviruses. In addition to the basic functions encoded by *gag*, *pol*, and *env*, the HIV-1 genome includes at least six additional genes with distinct regulatory roles (see references 31 and 39 for reviews). Two of these regulatory genes, *tat* and *rev*, are essential for virus gene expression. The remaining genes, *vpr*, *vpu*, *vif*, and *nef* are dispensable for virus replication in tissue culture, but mutations of these genes alter the replication properties of the virus.

HIV-1 *vpr* encodes a protein (viral protein R [Vpr]) of 96 amino acids (27). Previous studies have shown that the *vpr* products can increase the rate of replication of the virus and accelerate its cytopathic effects in T-cell lines and in peripheral blood mononuclear cells (PBMCs) (6, 8, 28, 29). Cohen and colleagues suggested that *vpr* increased gene expression from the HIV-1 promoter, as well as a wide range of other promoters, but the mechanism of this effect remains to be determined (7). *vpr* is also found in the genomes of HIV-2 and several strains of simian immunodeficiency virus (SIV) (5, 14). The activity of the HIV-2 and SIV *vpr* gene products appears to be similar to that of HIV-1 *vpr* (16, 35). Furthermore, SIV$_{mac}$ *vpr* is important for the development of an AIDS-like disease in rhesus macaques (22).

HIV-1, HIV-2, and SIV *vpr* gene products have 26 to 36% amino acid identity (40). Certain features of the Vpr proteins among different HIV isolates are highly conserved, including the presence of a single cysteine residue at amino acid position 76 of HIV-1 Vpr, a predicted amphipathic alpha-helical loop in the N-terminal portion of the protein, and the presence of an arginine-rich carboxyl-terminal tail.

Vpr is packaged within the HIV-1 virion (6, 45). Similar findings have been reported for SIV$_{mac}$ Vpr (44). The Vpr protein is the only regulatory product of HIV-1 found in virus particles, though the homologous *vpx* gene products of HIV-2 and SIV$_{mac}$ are also associated with virus particles (17–19). However, the subcellular distribution of Vpr and the mecha-

nism of incorporation into virus particles are unclear. In this study, the subcellular localization of Vpr in HIV-1-infected PBMCs and in two different *vpr* expression systems in mammalian cells was examined by subcellular fractionation and indirect immunofluorescence techniques. The role in cellular localization of the carboxyl-terminal arginine-rich sequence of Vpr was specifically studied. Lastly, the effects of Gag coexpression on Vpr export and incorporation into virus particles were examined.

MATERIALS AND METHODS

Cell lines and culture. COS-7 cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 1 mM pyruvate, 100 U of penicillin per ml, and 100 µg of streptomycin per ml. BSC40 cells were maintained in the same medium. PBMCs were purified from normal human leukocytes by centrifugation onto Ficoll. After 3 days of stimulation with phytohemagglutinin (15 µg/ml; Sigma), PBMCs were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 4 mM glutamine, 50 U of recombinant interleukin 2 (Cetus) per ml, 100 U of penicillin per ml, and 100 µg of streptomycin per ml.

Vpr and Gag expression plasmids. HIV-1 nucleotides 5558 to 5869 (numbered according to reference 27) encompassing the pNL4-3 *vpr* gene was obtained by the polymerase chain amplification reaction, using primers AATACCATGGAA CAAGCCCCAGAAGA and GATGCTTCCAGGGATCCGT CTAGGATCTACTG. The reaction product was digested with *Nco*I and *Bam*HI and cloned into pTM3 (designated here pTM) (12, 26), to produce pTM-VPR. The *Nco*I-*Bam*HI fragment of pTM-VPR was cloned between the *Sal*I and *Sac*I sites of pSRalpha (25) after blunt ending with T4 DNA polymerase, in the correct orientation (pSR-VPRs) and in the incorrect or antisense orientation (pSR-VPRa) (see Fig. 2A). The CRST mutant clone was constructed by digestion of pNL4-3 with *Sal*I, at nucleotide 5786, and blunt ending with the Klenow fragment of DNA polymerase I. It was then cloned

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into the pTM vector by the strategy used to construct pTM-VPR.

An NcoI-NcoI fragment from plasmid pGG1 (3, 32), containing nucleotides 789 to 5674, was cloned into the NcoI site of plasmid pTM3, to produce plasmid pTM-GAG-POL. This clone contains the gag and pol open reading frames. Expression of the pol gene was abrogated by frameshift mutation at the BclI site at nucleotide 2428 in the 5' portion of pol to produce pTM-GAG. Plasmid pTM-GAG(p41) was constructed from pTM-GAG-POL by frameshift mutation at the ApaI site at nucleotide 2005, using T4 DNA polymerase I. This results in a termination codon at nucleotide 2058 after the first Cys-His box coding region of p9.

Vpr and Gag p24 antisera. A New Zealand White rabbit was inoculated with complete Freund's adjuvant containing 200 μg of Vpr protein, synthesized according to the sequence of HIV-1 strain LAI and kindly provided by H. Gras-Masse (13). Booster doses of 200 μg of Vpr in incomplete Freund's adjuvant were given at 3, 6, 9, and 18 weeks after the initial inoculation. A New Zealand White rabbit was inoculated with complete Freund's adjuvant containing 100 μg of recombinant p24 protein (provided by American Biotechnology through the NIH AIDS Research and Reference Reagent Program), and 100-μg booster doses were given 2 and 4 weeks later.

Virus infection. HIV-1 virus stocks were generated by transfection of 60% confluent 10-cm-diameter COS-7 plates with 10 μg of recombinant proviral clone NLHXADA(GG) (41) and 2 μg of pCV1 (tat expression vector [1]) by the calcium phosphate precipitation method, followed 5 h later by 10% dimethyl sulfoxide shock for 2 min. The cells were washed twice with phosphate-buffered saline (PBS) before refeeding with 10 ml of fresh medium. Culture supernatants were harvested after 48 h and filtered (0.2-μm-pore-size Millipore filter). Five milliliters of culture supernatant was used to infect 5×10^7 PBMCs. Virus replication was monitored by determination of reverse transcriptase activity (30). PBMCs (10^7) were labeled for 20 h in 2 ml of leucine-free RPMI 1640 medium containing 200 μCi of [4,5-^{3}H]leucine and fractionated as described below. Mock-infected cultures were exposed to 5 ml of filtered culture supernatants from untransfected COS-7 cells.

Transfection and radiolabeling of COS-7 cells. COS-7 cells were grown to 60% confluence on 10-cm-diameter culture dishes and transfected with 15 μg of pSR-VPRs or pSR-VPRa by lipofection as recommended by GIBCO. Briefly, 15 μl of Lipofectin (GIBCO) was mixed with 3 ml of Opti-MEM I reduced-serum medium (GIBCO), and then 15 μg of DNA was added. The mixture was allowed to incubate at room temperature for 10 min before addition of the cells. Forty-eight hours after transfection, the cells were labeled with 4 ml of leucine-free DMEM containing 100 μCi of [4,5-^{3}H]leucine per ml for 40 h.

Infection-transfection protocol for the vaccinia virus expression system. BSC40 cells were grown to 90% confluence on 10-cm-diameter plates, infected for 1 h at 37°C with vTF7-3 (12, 26) at a multiplicity of infection of 10, and transfected with pTM vectors by the lipofectin transfection method. Four hours after transfection, the cells were labeled for 20 h with 3 ml of leucine-free DMEM containing 100 μCi of [4,5-^{3}H]leucine per ml.

Subcellular fractionation. Labeled cells were fractionated into membrane, cytosolic, postnuclear, and nuclear fractions as previously described (23), with minor modifications. Nuclei were further fractionated into nucleoplasm, chromatin extract, and nuclear matrix as described by Staufenbiel and Deppert (37). Cells were washed with ice-cold PBS and scraped in PBS.

The cell pellet volume was measured and resuspended in 10 volumes of Dounce buffer (10 mM Tris-HCl [pH 7.5], 1.5 mM MgCl$_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM dithiothreitol). The cells were allowed to swell on ice for 10 min before disruption with 20 to 25 strokes of a Dounce homogenizer. A small aliquot was saved and mixed with an equal volume of 0.4% (wt/vol) trypan blue in PBS to examine cell disruption under phase microscopy. Dounce homogenization was continued until >99% cells were disrupted. The homogenate was centrifuged at 1,500 rpm for 10 min in a Beckman GS-6 rotor to generate the supernatant containing both the membrane and cytosolic fractions and the nuclear pellet.

The nuclear pellet was subsequently extracted by four steps. First, the nuclear pellet was resuspended in buffer A (10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES-KOH; pH 7.4], 0.25 M sucrose, 0.2 mM PMSF, 0.5 mM dithiothreitol) supplemented with 0.1% (vol/vol) Triton X-100 and then incubated for 15 min on ice. The nuclei were pelleted at 1,500 rpm for 10 min, and the supernatant was designated the postnuclear wash fraction. Second, the pellet was resuspended in buffer A supplemented with 0.5% Nonidet P-40 and incubated for 30 min on ice. The nuclei were pelleted again at 1,500 rpm for 10 min, and the supernatant was designated the nucleoplasmic fraction. The latter procedure was repeated twice, and the supernatants were pooled. Third, the Nonidet P-40-extracted nuclear pellet was subjected to DNase I digestion (1% [vol/vol] Triton X-100, 1.5 mM MgCl$_2$, 0.2 mM PMSF, and 50 μg of DNase I [Sigma] per ml in PBS) for 15 min at 37°C. Then an equal volume of 4 M NaCl was added, and incubation was continued for 30 min at 4°C. The sample was then subjected to centrifugation at 2,500 rpm for 10 min. The supernatant was designated the chromatin extract, and the pellet was resuspended in radioimmunoprecipitation assay (RIPA) buffer (1% [vol/vol] Triton X-100, 0.5% [wt/vol] deoxycholate, 0.1% [wt/vol] sodium dodecyl sulfate [SDS], and 0.2 mM PMSF in PBS) for 30 min on ice. The insoluble portion was removed by centrifugation at 1,000 rpm for 15 min. The supernatant was designated the nuclear matrix. The purity of the nuclei was examined by using a control cytosolic protein, β-galactosidase, expressed in the same cells by transfection of a cDNA expression clone. More than 98% of the β-galactosidase activity was found in the cytosol, as measured by enzymatic assay. Only 1.4% of the β-galactosidase activity was detected in the postnuclear wash fraction. No detectable activity was found in the purified nuclei.

For the membrane and cytosolic fractions, the salt concentration was adjusted to 0.15 M NaCl and then the preparations were fractionated by ultracentrifugation at 100,000 × g for 30 min. The supernatant was designated the cytosolic fraction. The pellet was washed with 1 M NaCl in PBS for 30 min on ice, and ultracentrifugation was repeated. The supernatant was designated the membrane wash fraction, and the membrane pellet was resuspended in RIPA buffer.

Immunoprecipitation. Equivalent proportions (volume/volume) of each of the subcellular fractions were precipitated overnight with 10% trichloroacetic acid at 4°C. The resulting pellets were washed in 70% ethanol, solubilized in sample buffer (0.125 M Tris-HCl [pH 6.8], 20% glycerol, 10% [vol/vol] 2-mercaptoethanol, 4% [wt/vol] SDS). An aliquot was taken for scintillation counting to determine relative labeled protein content.

Equivalent proportions (volume/volume) of each of the subcellular fractions were adjusted to 500 μl of RIPA buffer. Immunoprecipitation was performed by the addition to 500 μl of each fraction 5 μl of anti-Vpr antiserum or 5 μl of anti-Gag

antiserum. Incubation was continued overnight at 4°C. Twenty microliters of protein A-Sepharose beads (50% [vol/vol] in PBS) was added, and the mixture was incubated for 120 min at 4°C. Immunoprecipitates were collected at 500 × g for 3 min at room temperature and washed three times with RIPA buffer. The beads were resuspended in 30 μl of sample buffer. Samples were treated at 100°C for 10 min before SDS–12% polyacrylamide gel electrophoresis (PAGE), fixation for 30 min in 25% isopropanol–10% acetic acid, treatment with Amplify (Amersham), and autoradiography were performed. Band intensities were determined by densitometry.

Immunofluorescence. BSC40 cells (10^4) were plated on eight-well Lab-Tek chamber slides overnight. The cells were infected with vTF7-3 and transfected as described above. The cells were fixed with 2.5% (wt/vol) glutaraldehyde for 15 min and permeabilized with 0.2% (vol/vol) Triton X-100 for 6 min at room temperature. The cells were then blocked for nonspecific binding of immunoglobulin by incubation for 30 min with PBS containing 5% (wt/vol) nonfat dry milk and 0.1% (vol/vol) Tween 20. Slides were then incubated with rabbit anti-Vpr antibody (1:100 in Tween buffer [PBS with 0.5% Tween 20 and 1% bovine serum albumin]) and mouse monoclonal antihistone antibody (1:500 in Tween buffer; Chemicon) for 1 h at room temperature. The cells were washed several times with 0.3% (vol/vol) Triton X-100 in PBS and incubated at 4°C for 60 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G to detect Vpr and rhodamine-conjugated goat anti-mouse immunoglobulin G to detect histones. The slides were washed extensively with PBS and mounted in Aqua mount solution (Lerner Lab) containing 2.5% 1,4-diazobicyclo-[2,2,2]-octane (Sigma) to prevent photobleaching of the FITC signal. Slide preparations were examined on a Nikon fluorescence microscope equipped with appropriate filters and a microflex UFX camera system. Photographs were prepared by using Kodak T-MAX film, push processed to ASA 3200.

Sucrose gradients. BSC40 cells were infected, transfected, and labeled with [^{3}H]leucine as described above. Cellular debris was removed from the conditioned medium by centrifugation at 2,500 rpm for 15 min in a Beckman GS-6 rotor. Particles were concentrated by sedimentation through a 20% sucrose cushion prepared in PBS at 28,000 rpm for 90 min at 4°C in an SW28.1 rotor. Particles were resuspended in 200 μl of PBS, layered on a linear 20 to 60% sucrose gradient in PBS, and centrifuged in an SW28.1 rotor at 20,000 rpm for 16 h at 4°C. Fractions were collected from the top of the tube.

RESULTS

Localization of Vpr in HIV-1-infected PBMCs. To examine the intracellular localization of Vpr, PBMCs, a natural target cell population, were chosen for HIV-1 infection. HIV-1 strain NLHXADA(GG) was chosen since it encodes a functional 96-amino-acid form of Vpr identical in amino acid sequence to that encoded by NL4-3 (27, 29, 42). Nine days after infection, the cells were labeled for 20 h with [^{3}H]leucine, lysed by Dounce homogenization, and then fractionated into nuclear, cytosolic, and membrane fractions by differential centrifugation. Each fraction was immunoprecipitated with a polyclonal rabbit anti-Vpr antiserum and subjected to SDS-PAGE (Fig. 1). A Vpr-specific protein of 14 kDa was detected in NLHX ADA(GG)-infected cells and conditioned medium but not in mock-infected cultures. This protein was not immunoprecipitated with a control antiserum obtained from the prebleed serum of the same rabbit prior to inoculation with the synthetic
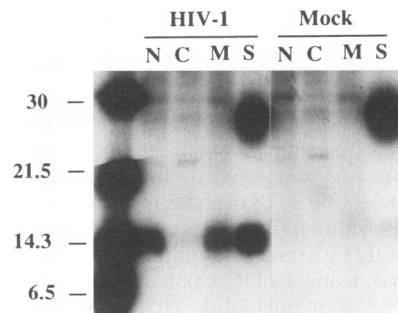

FIG. 1. Localization in PBMCs of Vpr expressed from infectious virus. PBMCs were infected for 9 days with HIV-1 strain NLHX ADA(GG) or were mock infected. The cells were labeled with [^{3}H]leucine, the medium was harvested (S), and the cells were fractionated into nuclear (N), cytosolic (C), and membrane (M) fractions as described in Materials and Methods. Equivalent portions of each fraction were immunoprecipitated with the anti-Vpr antiserum and analyzed by SDS-PAGE. Molecular mass markers are shown at the left in kilodaltons.

Vpr used for production of the anti-Vpr antiserum (not shown).

The majority of Vpr was found in the culture supernatant, consistent with previous reports that Vpr is virion associated (6, 45). Intracellular Vpr was found in both nuclear (26%) and membrane (20%) fractions. Vpr in the nuclear fraction was not due to contamination with unbroken cells, which made up <1% of the total cell population used in the fractionation experiments. Less than 4% of the Vpr was found in the cytosolic fraction. Approximately 50% of the labeled Vpr was found in the conditioned medium.

Expression and localization of Vpr in mammalian cells. To study the cellular localization of Vpr without the effect of other HIV-1 components, the NLHXADA(GG) *vpr* gene was cloned into a simian virus 40 (SV40)-based vector system, pSRalpha, in both the correct (pSR-VPRs) and incorrect or antisense (pSR-VPRa) orientations (Fig. 2A). The expression plasmid contains both the SV40 early enhancer (SV40-ori) and a human T-cell leukemia virus type I (HTLV-I) promoter with R and U5 elements of the HTLV-I long terminal repeat. This expression plasmid has previously been reported to achieve high levels of expression of a number of different lymphokine cDNAs in a variety of cell types (38) and to facilitate the expression of HIV-2 *vpx* in COS-1 cells (21).

pSR-VPRs and pSR-VPRa were transfected into COS-7 cells, and the cells were labeled with [^{3}H]leucine and separated into membrane, membrane wash, cytosolic, and nuclear fractions. Membranes loosely associated with nuclei were removed by a wash with 0.1% Triton X-100 and were designated the postnuclear wash. Soluble nucleoplasmic proteins were extracted with two successive washes in 0.5% Nonidet P-40, which permeabilizes the nuclear membrane (2). This method has previously been demonstrated to preserve overall nuclear and nucleolar architecture (33). The chromatin fraction was obtained by digestion of the resultant insoluble nuclear fraction with DNase I and by a subsequent wash in a high-salt buffer. This fraction contained all of the major histone proteins found in intact nuclei (not shown). The salt- and detergent-insoluble fraction was pelleted to yield the nuclear matrix fraction, which was solubilized in RIPA buffer.

The partition of Vpr during fractionation was examined by immunoprecipitation with the anti-Vpr antibody, SDS-PAGE, and densitometric quantitation (Fig. 2B). The chromatin frac-
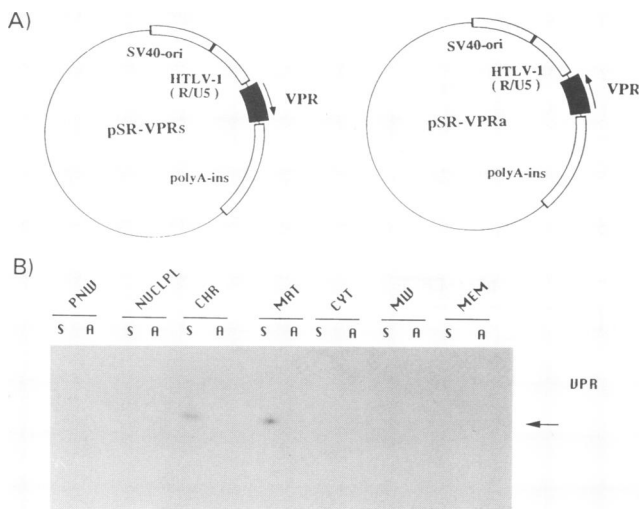
A)

B)

FIG. 2. Localization in COS-7 cells of Vpr expressed from pSR. (A) Vpr sense (pSR-VPRs) and antisense (pSR-VPRa) expression plasmids, which include a transcriptional enhancer (SV40-ori), a transcriptional promoter (HTLV-I long terminal repeat [R/U5]), and a polyadenylation insertion sequence (polyA-ins). (B) Subcellular fractionation of Vpr expressed in transfected and [³H]leucine-labeled COS-7 cells from pSV-VPRs (S) and pSR-VPRa (A) in postnuclear wash (PNW), nucleoplasm (NUCLPL), chromatin (CHR), nuclear matrix (MAT), cytosol (CYT), membrane wash (MW), and membranes (MEM). Equivalent amounts of each fraction were immunoprecipitated with the anti-Vpr antiserum and analyzed by SDS-PAGE. The electrophoretic position of Vpr is shown by an arrow at the right.

tion included 17% of the intracellular labeled proteins and 44% of the total Vpr. The nuclear matrix included 1% of the intracellular labeled proteins and 56% of the total Vpr. Less than 1% of the total Vpr was found in the other cellular fractions.

Truncation of the C terminus of Vpr impairs nuclear localization. Most nuclear localization signals consist of a short stretch of positively charged amino acids (15). Interestingly, the C terminus of Vpr contains a high proportion of positively charged amino acids, including 7 arginine residues among the C-terminal 20 amino acids (Fig. 3A). To characterize the role of this C-terminal sequence, a vaccinia virus expression system was used to achieve high-level and rapid expression of Vpr. The NLHXADA(GG) *vpr* gene was cloned into pTM3, a plasmid utilizing a T7 promoter for heterologous gene expression. This plasmid was designated pTM-VPR. A carboxyl-terminal truncation mutant of pTM-VPR, pTM-CRST, was constructed by frameshift mutation at the *Sal*I site. A recombinant vaccinia virus, vTF7-3, which encodes T7 RNA polymerase was used for expression in mammalian cells.

BSC40 cells were infected with vTF7-3 and transfected with pTM-VPR or pTM-CRST. Cells were labeled with [³H]leucine, cell supernatants were harvested, and disrupted cells were fractionated into membrane, membrane wash, cytosolic, and postnuclear wash fractions and various nuclear fractions (nucleoplasmic proteins, chromatin, and nuclear matrix). Equivalent amounts of each fraction were immunoprecipitated with the anti-Vpr antiserum and analyzed by SDS-PAGE (Fig. 3B), and band intensities were quantitated by laser densitometry (Fig. 3C). The predominant Vpr product expressed from pTM-VPR had an electrophoretic mobility of a 14-kDa protein, with a minor band with a mobility of a 13-kDa protein. Vpr expressed from pTM-CRST electrophoresed as a 12-kDa

protein, consistent with the removal of 17 amino acids from the carboxyl terminus.

Eighty-four percent of pTM-VPR-expressed Vpr was found in nuclear fractions, primarily the nuclear matrix and chromatin fractions (Fig. 3B [left] and C). This result is in agreement with the fractionation data with pSR-VPR-expressed Vpr (Fig. 2). Eight percent of pTM-VPR-expressed Vpr was tightly associated with the membrane fraction (Fig. 3B and C, MEM). The possible discrepancy in the amount of membrane association of Vpr expressed with the vaccinia virus expression system compared with the data obtained with pSR-VPR (Fig. 2) may be related to the significantly higher level of expression of Vpr with the vaccinia virus expression system than with the SV40 plasmid expression system. Only 4% of Vpr was found in the cytosol (Fig. 3B and C, CYT), and no detectable Vpr was released from cells into the cell supernatant.

Deletion of the arginine-rich C terminus of Vpr resulted in a dramatic shift of Vpr cellular localization (Fig. 3B [right] and C). Only 25% of the truncated Vpr was retained in the nuclear fraction. Furthermore, the distribution in nuclear fractions of pTM-CRST product was distinctly different from that of pTM-VPR, with the majority of the truncated protein in the nucleoplasm. Twenty-four percent of the pTM-CRST protein was in the postnuclear wash, compared with 3% of the pTM-VPR product. Thirty-eight percent of the mutant Vpr was found in the cytosol, compared with 4% of the parental Vpr. Similar amounts of pTM-CRST and pTM-VPR products were bound to membranes.

Indirect immunofluorescence localization of Vpr. Subcellular fractionation experiments indicated predominant localization of Vpr in the nucleus. To confirm these results, indirect immunofluorescence was performed with fixed cells. BSC40 cells were infected with vTF7-3 and then transfected with pTM-VPR or pTM. Vpr was detected by anti-Vpr rabbit antibody and visualized with FITC-conjugated anti-rabbit antibody. Intense immunofluorescence was observed in the majority of cells transfected with pTM-VPR (Fig. 4A, left), but no fluorescence was observed in cells transfected with the vector pTM alone (Fig. 4A, right) or if preimmune serum was used (not shown).

Four types of staining patterns were observed in four independent experiments in which 50 cells were randomly selected and enumerated. Sixty-two percent of the cells showed a diffuse nuclear and focal perinuclear staining pattern (Fig. 4B, middle). The nucleus is visualized by phase-contrast microscopy (Fig. 4B, left) and mouse antihistone and rhodamine-conjugated anti-mouse antibody (Fig. 4B, right). Twenty-seven percent of the cells showed focal perinuclear staining only. Six percent of the cells had diffuse perinuclear staining with intense immunofluorescence surrounding the nucleus. Four percent of the cells showed only diffuse nuclear staining.

Influence of Gag protein on Vpr export and virion incorporation. In HIV-1-infected cells, Vpr was found to be exported into the medium in virus particles (Fig. 1) (6, 45), though no export was found when Vpr was expressed in the absence of other virion components (Fig. 3). To assess the requirements for export, Vpr was coexpressed with the HIV-1 Gag p55 precursor protein by using plasmid pTM-GAG. BSC40 cells were infected with vTF7-3 and transfected with pTM-VPR alone, pTM-GAG alone, or both plasmids. No differences were noted in the electrophoretic mobility or quantity of the 14-kDa product in the cell lysates with pTM-VPR expressed in the presence or absence of pTM-GAG (Fig. 5A, left). The pTM-GAG product was primarily a 55-kDa protein, with smaller amounts of 43- and 41-kDa products. The latter proteins were found to be Gag proteins, since they did not react with a
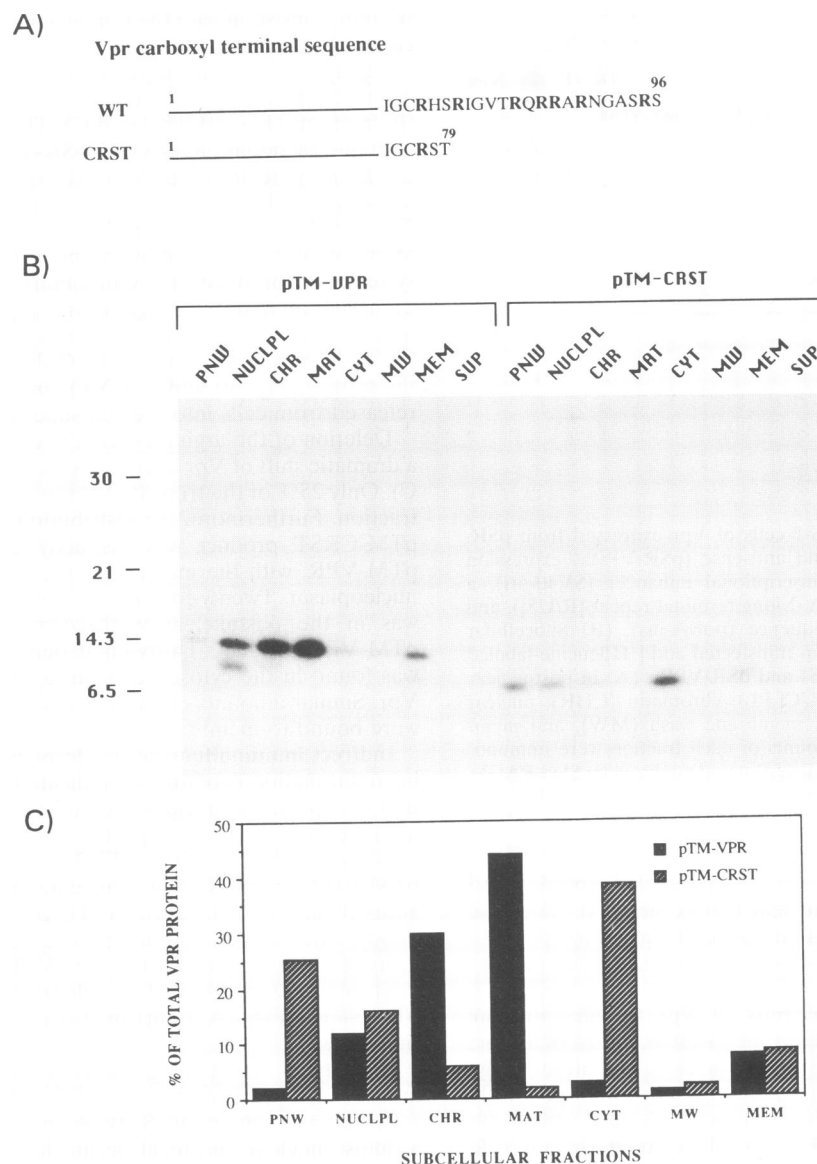
FIG. 3. Subcellular distribution of vaccinia virus-expressed parental Vpr and carboxyl-terminal truncation mutant CRST in BSC40 cells. (A) Schematic drawing of the Vpr protein, indicating the carboxyl-terminal arginine-rich sequence of the wild type (WT) and of the truncation mutant, CRST. (B) Vpr expressed from pTM3 in vTF7-3-infected cells. Cells were labeled with [^{3}H]leucine and fractionated into postnuclear wash (PNW), nucleoplasm (NUCLPL), chromatin (CHR), nuclear matrix (MAT), cytosol (CYT), membrane wash (MW), membranes (MEM), and cellular supernatant (SUP). Molecular mass markers are shown at the left in kilodaltons. (C) Proportion of VPR in each subcellular fraction as determined by laser densitometry from pTM-VPR (solid bars)- or pTM-CRST (hatched bars)-transfected cells.

preimmune serum or the anti-Vpr antibody (not shown). These smaller proteins may represent nonspecific cleavage products, products from initiation at a downstream AUG codon, or premature translational termination. No effects on Gag protein expression were noted with coexpression of Vpr.

Expression of pTM-VPR alone did not result in export in the cell supernatant (Fig. 5A, right). Expression of pTM-GAG resulted in the 55-kDa product in the cell supernatant. Coexpression of pTM-GAG with pTM-VPR promoted the export of Vpr into the cell supernatant.

To determine whether the viral proteins released into the cell supernatant were associated with particles, sucrose gradient analysis was performed (Fig. 6). Particles were first con-

centrated from the cell supernatant samples by centrifugation through a 20% sucrose cushion. The resultant particulate material was resuspended and analyzed on a linear 20 to 60% sucrose gradient. Each fraction was concentrated with 10% trichloroacetic acid and analyzed by SDS-PAGE. No particle-associated protein was found from cells transfected with pTM-VPR alone (Fig. 6A). Expression of pTM-GAG alone resulted in particle-associated Gag protein banding in fractions 10 and 11, at a density of 1.16 to 1.17 g/ml (Fig. 6B). Expression of pTM-GAG together with pTM-VPR resulted in cosedimentation of both Vpr and Gag in fractions 11 and 12, at a density of 1.16 to 1.17 g/ml (Fig. 6C).

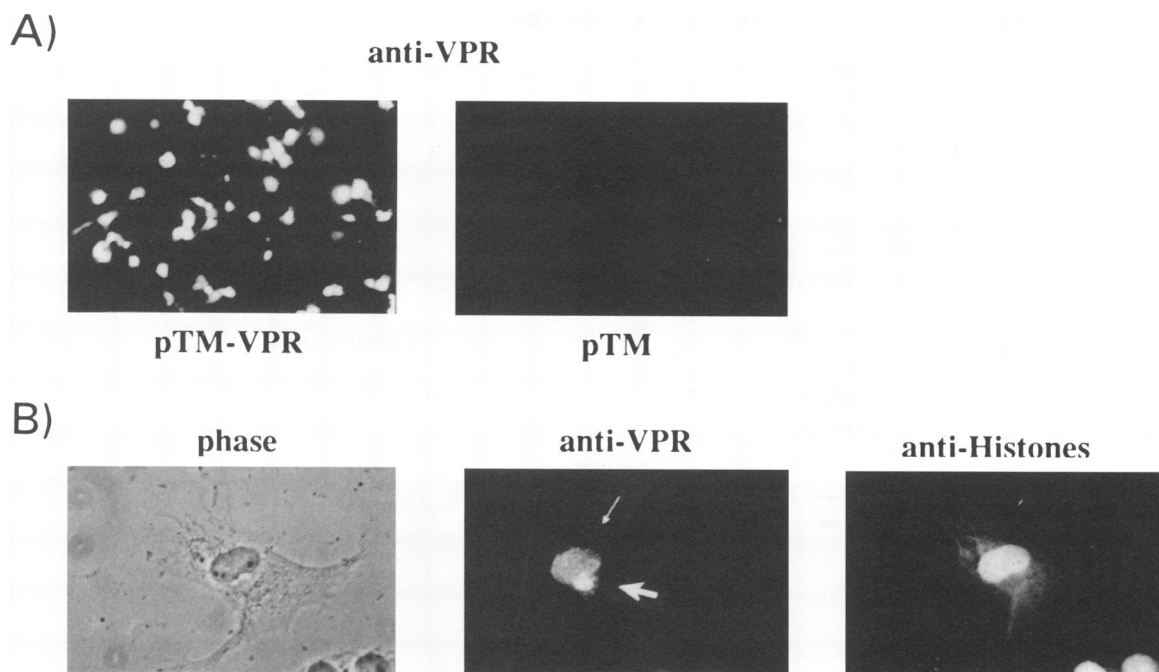Vpr packaging was also assessed with a clone expressing a

FIG. 4. Immunofluorescence localization of Vpr in BSC40 cells. (A) pTM-VPR (left)- or pTM (right)-transfected vTF7-3 infected cells were incubated with the anti-Vpr antiserum and an FITC-conjugated goat anti-rabbit immunoglobulin. Magnification, ×188. (B) Higher magnification (×752) by phase-contrast microscopy (left) and fluorescence microscopy (middle and right) of a representative cell incubated with anti-Vpr antiserum and FITC-conjugated goat anti-rabbit immunoglobulin (middle; filter with excitation range of 450 to 490 nm and emission range of 520 to 560 nm) and antihistone antiserum and rhodamine-conjugated goat anti-mouse immunoglobulin (right; filter with excitation range of 510 to 560 nm and emission range of >590 nm). Diffuse nuclear (thin arrow) and focal perinuclear staining (thick arrow) are indicated in the middle panel.

truncated form of the Gag precursor protein, pTM-GAG(p41), in which all of the amino acids following the first Cys-His box of NC as well as the C-terminal p6 coding sequence were removed. This construct has been shown to produce virus-like particles in the vaccinia virus infection-transfection system in a manner similar to that of pTM-GAG (not shown). When coexpressed with pTM-VPR, p41 appeared in the supernatant but did not result in the export of Vpr from the cells (Fig. 5B). The failure to detect Vpr in the cell supernatant was due to the absence of Vpr export rather than to the lower quantity of p41 Gag particles produced, since no Vpr was detected, even after overexposure of the autoradiogram shown in Fig. 5B. In contrast, in the same experiment, production of p55 from pTM-GAG resulted in significant export of Vpr.

DISCUSSION

Localization of Vpr in the nucleus. In this study, we used three different expression systems to provide evidence for the localization of a significant proportion of Vpr in the nucleus, as demonstrated by subcellular fractionation techniques. In HIV-1-infected PBMCs, 26% of the expressed Vpr was found in the nucleus (Fig. 1). In contrast, when Vpr was expressed in the absence of other viral components by using an SV40 expression plasmid, almost all of the protein was found in the nucleus (Fig. 2). Similar results were obtained with the vaccinia virus expression system, in which case 84% of the Vpr was found in the nucleus (Fig. 3). Results of the indirect immunofluorescence experiments support the results obtained by using subcellular fractionation techniques, indicating nuclear staining in 66% of Vpr-expressing cells (Fig. 4; see Results).

Further fractionation of the isolated nuclei provides additional evidence for Vpr localization in the nucleus rather than in membranes loosely associated with the nuclear membranes. These experiments identified the predominant association of Vpr with the chromatin and nuclear matrix fractions (Fig. 2 and 3). The association of Vpr with the nuclear matrix is unlikely to be spurious, since it is resistant to Nonidet P-40, DNase, and high-salt extraction procedures. Although the role of the nuclear matrix in transcriptional regulation is unclear, several studies have indicated that it may play an important role. The nuclear matrix has been reported to have a role in mRNA transcription and processing via its involvement in attachment and/or association with newly transcribed mRNA (20), ribonucleoprotein particles (11), and pre-mRNA splicing machinery (36, 46). Several gene products, characterized for their ability to promote oncogenic transformation, are also associated with the nuclear matrix. These include the large T antigen of polyomavirus (4), myc gene products (9), the adenovirus E1A protein (10), and the Tax protein of HTLV-I (43). The presence of Vpr in the nuclear matrix might indicate a role in trans activation of viral gene expression or RNA processing. This is consistent with a report by Cohen and colleagues suggested that Vpr may serve as a trans activator of HIV-1 gene expression as well as a trans activator of other genes (7). However, the mechanism of this effect and its relevance to Vpr action during virus replication remain unclear. Alternatively, Vpr association with the nuclear matrix may affect host cell gene expression. This view is consistent with a recent report that Vpr induces muscle cell differentiation (24).

Though Vpr lacks a classical nuclear localization signal (15), the carboxyl-terminal portion of the protein is rich in basic amino acids. A truncation mutation which removes the carboxyl-terminal 19 amino acids was found to impair Vpr localiza-
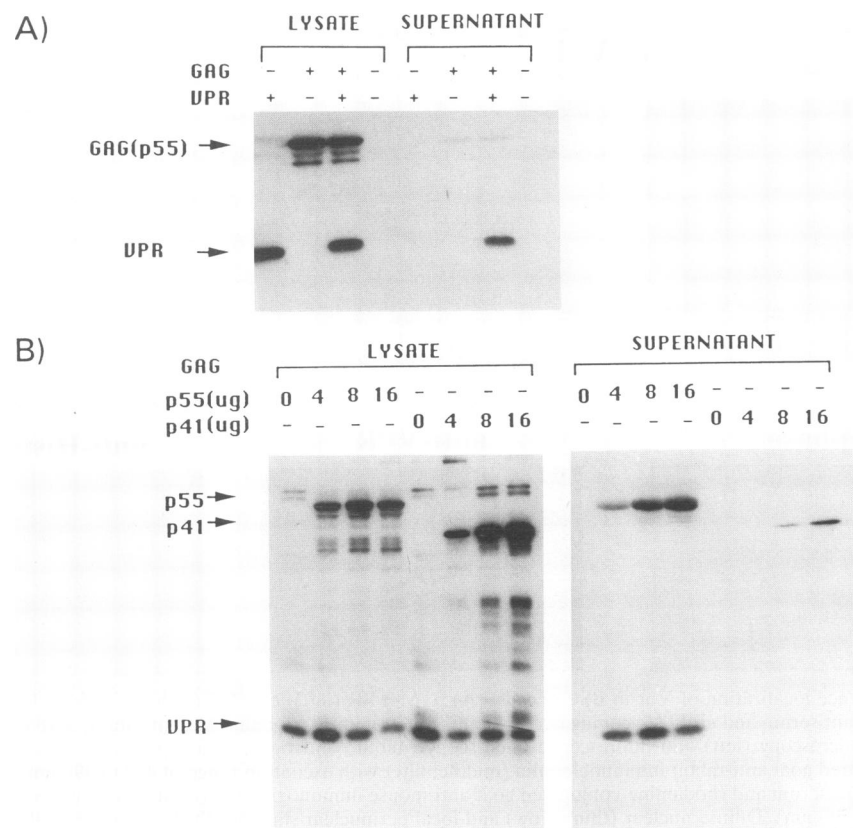
FIG. 5. Influence of Gag expression on Vpr localization. (A) BSC40 cells (10^6) were grown on 60-mm-diameter plates overnight and then infected with vTF7-3 and transfected with 7.5 μg of pTM-VPR (VPR) and/or 7.5 μg of pTM-GAG (GAG). The negative control cells were transfected with 15 μg of pTM vector. The cells were labeled with 100 μCi of [^{3}H]leucine per ml for 16 h, scraped in PBS, and resuspended in RIPA buffer. The cell lysates and cell supernatant fractions were immunoprecipitated with both the anti-Vpr and anti-Gag antisera and analyzed by SDS-PAGE. The electrophoretic positions of Gag and Vpr are shown at the left. (B) BSC40 cells (0.3×10^6) on 35-mm-diameter plates were infected and transfected with 2 μg of pTM-VPR and 0, 4, 8, or 16 μg of pTM-GAG(p55) or pTM-GAG(p41), as indicated at the top. The cells were labeled and analyzed as described above.

tion in the nucleus (Fig. 3). Furthermore, the distribution of the small proportion of truncated Vpr found in the nucleus was distinctly different from that of full-length Vpr, with the truncated Vpr localized predominantly in the nucleoplasm and very little Vpr in the nuclear matrix or chromatin fractions. It is possible that truncation of the carboxyl-terminal portion of Vpr alters the conformation of the molecule. Alternatively, it is possible that the carboxyl-terminal arginine-rich sequence serves as at least part of a nuclear localization signal. This view is supported by our preliminary observations that attachment of the C-terminal 19-amino-acid Vpr sequence onto β-galactosidase directs this protein to the nucleus (not shown).

Previous studies with lymphoid cells had indicated an important functional role for the C-terminal Vpr sequence (29). Therefore, these findings are consistent with an important role for Vpr localization in the nucleus for HIV-1 replication.

Membrane-associated Vpr. Though very little Vpr could be identified in the cytosol, a small proportion was consistently associated with the membrane fraction. This observation is in agreement with findings of Sato and colleagues (34). In HIV-1-infected PBMCs, 20% of Vpr was found in the membrane fraction (Fig. 1), whereas with the vaccinia virus expression form of the protein, 8% was found in the membrane (Fig. 3B and C). The indirect immunofluorescence experiments also suggested that some Vpr is found at an extranuclear site but

closely associated with the nucleus (Fig. 4; see Results). The latter site may represent intracellular membranes, possibly with either the endoplasmic reticulum or Golgi apparatus. However, a Golgi location for Vpr is unlikely, since brefeldin A treatment did not change Vpr localization (not shown). The nature and significance of membrane localization of VPR require further analysis.

VPR export from cells and incorporation into virus particles. Several previous studies have demonstrated that HIV- and SIV-expressed Vpr is incorporated into virus particles (6, 44, 45). This finding is in agreement with our observation that 50% of Vpr expressed in HIV-1-infected PBMCs is exported from the cells (Fig. 1). Vpr expression in the absence of other viral components resulted in no detectable export (Fig. 3 and 5). However, coexpression with the Gag p55 precursor protein resulted in export of VPR from the transfected cells (Fig. 5) and incorporation into virus-like particles (Fig. 6). Thus, Vpr incorporation into virus particles is independent of viral envelope incorporation. This finding suggests that Vpr associates directly or indirectly with a portion of the Gag precursor protein. The finding that the p41 truncation form of Gag is unable to package Vpr suggests the possibility of an interaction between either the p9 nucleocapsid protein or the proline-rich p6 protein and Vpr.

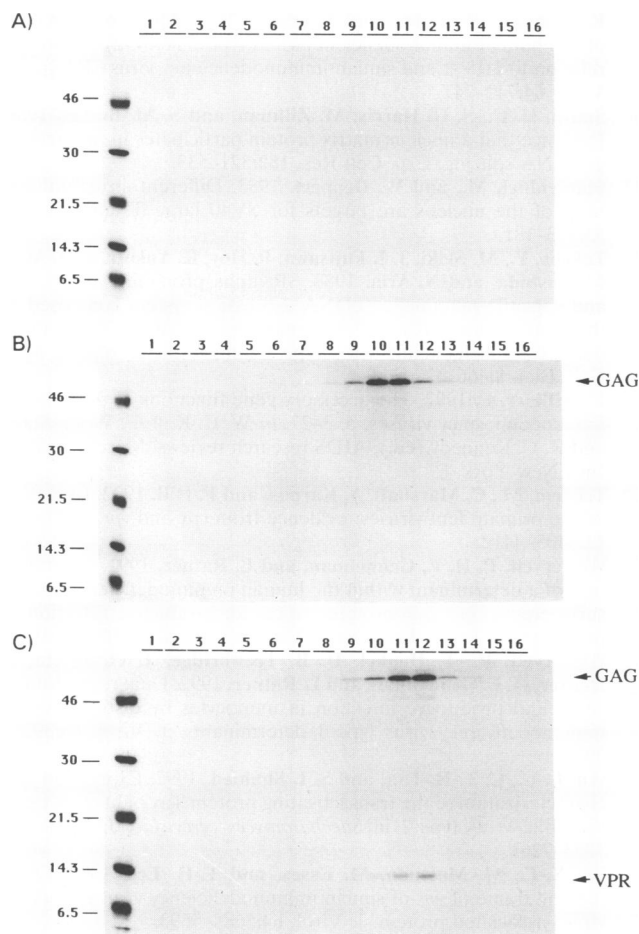Although the significance of Vpr incorporation into virions

FIG. 6. Sucrose gradient analysis of particles released from BSC40 cells transfected with pTM-VPR (A), pTM-GAG (B), pTM-VPR and pTM-GAG (C). Fractions were precipitated with 10% (wt/vol) trichloroacetic acid and analyzed by SDS-PAGE. Fraction 1 is from the bottom and fraction 16 from the top of each gradient. Molecular mass markers are shown at the left in kilodaltons.

is unclear, it is likely that this protein plays an important role in early events in the virus life cycle. It is tempting to speculate that the nuclear localization domain of Vpr allows targeting of the viral preintegration complex to the nucleus. Further studies on this important regulatory protein will be required to fully elucidate its role in the HIV life cycle.

ACKNOWLEDGMENTS

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